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(54) Title: LIPOPROTEIN CHOLESTEROL ASSAYS

(57) Abstract

The present invention relates to an assay procedure and/or device for directly measuring VLDL, LDL, HDL as well as apolipoproteins cholesterol in blood plasma and body fluids. The invention provides a method for direct quantitative determination of VLDL, LDL, HDL as well as apolipoproteins cholesterol in a sample of body fluid in which an antibody against the lipoprotein or apolipoprotein is partitioned in one of two phases of an aqueous two-phase system with or without the help of a partitioning enhancer by conjugation. A sample of body fluid, such as blood plasma, is applied to the aqueous two-phase system. After mixing and incubation for a short period of time, the phases are separated by gravity or centrifugation within several minutes. The phase containing the antibody and the targeted lipoprotein or apolipoprotein is removed. The targeted lipoprotein cholesterol is then directly determined by using an enzymatic procedure.

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Lipoprotein Cholest rol Assays

BACKGROUND OF THE INVENTION

The present invention relates to the clinical assay techniques for lipoprotein cholesterol determination.

Abnormal concentration of plasma lipoprotein is a major risk factor in the development of coronary heart disease. Plasma lipoproteins serve to solubilize and transport cholesterol and triglyceride in the blood stream, in which aqueous insoluble lipids bind to protein forming lipid-protein complexes that become soluble. A lipoprotein may be visualized as a spherical particle with an outer solubilizing coat of protein, phospholipid and free cholesterol and an inner hydrophilic neutral core of triglyceride and esterified cholesterol. There are four major classes of lipoproteins: chylomicrons, very-low density lipoprotein (VLDL) (density <1.006 gm/ml), low density lipoprotein (LDL) (density, 1.006-1.063 gm/ml), high density lipoprotein (HDL) (density > 1.063 gm/ml). These differ in their compositions, protein and lipid ratio, the latter determining the density of lipoprotein.

For epidemiologic and clinical purpose, it is convenient to measure their lipoproteins by quantifying the cholesterol moiety rather than their total mass. Increased total blood cholesterol level, especially, an elevated LDL cholesterol correlates with risk of coronary heart disease (CHD), and HDL cholesterol level is an inverse risk factor. There is another class of lipoprotein Lp (a) which is cholesterol rich for developing premature CHD. Even within the normal range of total cholesterol, higher LDL cholesterol is associated with increased occurrence of CHD. Reduction of elevated LDL cholesterol level is associated with a reduction in the incidence of cardiovascular disease and death in adults.

The National Cholesterol Education Program (NCEP) has made LDL cholesterol the basis for the classification and treatment of hypercholesterolemia. NCEP recommends that the risk of CHD may be classified as: for total chol sterol, desirable, <200 mg/dl; borderline-high risk, 200-239 mg/dl, high-risk, >240

mg/dl; for LDL cholesterol, desirable, <130 mg/dl, bord rline-high risk, 130-159 mg/dl, high risk, >160 mg/dl. That leads to an increased demand for accurate, simple, cost-effective LDL cholesterol and HDL cholesterol measurements. Total cholesterol can serve as a first step for prediction of CHD risk and its measurement is reasonably advanced and reference system is essentially complete. However, although there are a number of different methodologies available for LDL and HDL cholesterol measurements, no one has so far provided a useful tool for routine laboratory diagnosis. The purpose of this invention is to disclose a simple, cost-effective and easy to operate approach for routine lipoprotein cholesterol measurements.

There are several methods for cholesterol measurement currently available which will be discussed in detail below.

1) Sequential Differential Ultracentrifugation:

Lipoproteins vary greatly in size, density, relative composition, and biological function. Ultracentrifugation separates lipoproteins according to their density. Plasma is centrifuged at its own density of 1.006 kg/l. The VLDL and chylomicrons float to the top after centrifugation. The second centrifugation is performed after adjustment to increase the density of 1.006 kg/l to 1.063 kg/l to float LDL. Then LDL cholesterol is measured. In this method, although LDL cholesterol may be measured directly, it is time-consuming and needs expensive instrument, i.e., ultracentrifuge. It cannot be used in routine work for large population studies.

2) Density-Gradient Ultracentrifugation:

Another ultracentrifugation method for lipoprotein separation is based on discontinuous density-gradient. With this technique, the lipoprotein may be separated in a single centrifugation step. Different density solutions are carefully placed into each tube along with the sample. After centrifugation to equilibrium, each of the lipoproteins will have migrated into its respective isopycnic density region. That too is time-consuming. It also needs expensiv equipm nt

and specialists to run them.

3) Chromatography:

Several chromatographic techniques for separation of lipoproteins have been described, and their cholesterol content have been subsequently determined. In those procedures, lipoproteins are separated based on their size. It is known that there is a high correlation between lipoprotein density and particle size, owing to their chemical composition and structure. Lipoprotein separated by chromatography is correlated with those separated by ultracentrifugation. Agarose column and high performance liquid chromatography (HPLC) with gel permeation column have been successfully used to separate LDL, VLDL, HDL, and recovery of each lipoprotein is high. In case of incomplete separation with one agarose column, multiple columns may be used. Nevertheless, the procedure is time-consuming and cumbersome. Compared to agarose chromatography, HPLC is relatively simple and rapid. However, expensive equipment is needed. Chromatography technique is only restricted to laboratory research and cannot be used for diagnosis purpose because of the complexity and the length of the procedure, and the need for special instruments.

4) Electrophoresis:

There have been several reports on separation of lipoproteins by electrophoresis, in which lipoproteins are separated according to their charge and size. Different kinds of electrophoresis techniques have been used, including paper, cellulose acetate, agarose and polyacrylamide. Recently, gradient gel electrophoresis and capillary electrophoresis have been developed for lipoprotein fractionation. However, electrophoresis techniques would not be suitable for large population studies because they are time-consuming and labor intensive.

5) Beta-Quantification:

This method is recommended by the Centers for Disease Control (CDC) as a reference for LDL cholesterol measurement. The procedure involves combinati n of ultracentrifugation and

chemical precipitation. It begins with the s paration of VLDL by ultracentrifugation at 1.006 gm/ml. HDL and LDL are then separated. Instead of by ultracentrifugation, heparin-manganese precipitation of density larger than 1.006 gm/ml fraction is applied, where LDL is precipitated and HDL left in the supernatant. After sedimentation of LDL, HDL cholesterol is measured. LDL is calculated as the difference in cholesterol between the density >1.006 gm/ml fraction and the HDL. This procedure still needs one step of ultracentrifugation.

6) Friedewald Estimation:

In routine practice, LDL cholesterol is mostly derived by Friedewald estimation. It requires three separate measurements to determine the total cholesterol, HDL cholesterol and total triglycerides. LDL cholesterol is estimated from the Friedewald formula as follows:

LDL Cholesterol = Total Cholesterol - (HDL Cholesterol + Triglyceride/5)

Each of these measurements could introduce a certain degree of distortion and could lead to imprecision and inaccuracies in LDL cholesterol determination. LDL cholesterol value is increasingly inaccurate at triglyceride levels above 200 mg/dl. The procedure is not reliable in hypertriglyceridemic states (>400 mg/dl) and requires fasting samples as well.

7) Precipitation Methods:

A variety of precipitation methods have been used which depend upon the use of polyanions and divalent cations.

The following three methods for selective chemical precipitation of LDL were shown in the literature:

a) LDL is precipitated by heparin at pH - 5.12, achieved by including sodium citrate buffer.

b) LDL is precipitated from serum by polyvinyl sulfate in the presence of EDTA and polyethylene glycol methylether.

c) LDL may be precipitated from serum by unspecified amphipathic polymers in imidazole buffer at pH

6.10.

LDL cholesterol is calculated as the differenc b tween total cholesterol and cholesterol in the supernate, or is measured directly after dissolving the precipitate. These precipitation methods exhibit the same shortcomings as does the Friedewald formula. They have been judged to be superfluous for laboratories that have accurate and precise assays for total cholesterol, triglycerides and HDL cholesterol.

The precipitation methods give inaccurate results when serum triglyceride values are high, and do not distinguish between Lp(a) and LDL. The chemical precipitation methods introduced to date do no appear to have substantial advantage over other estimations, other than the fact that LDL cholesterol may be obtained from two measurements rather than three.

8) Direct LDL Methods:

In one of the direct LDL methods, polyclonal antibodies are coated to latex beads which are used to adsorb HDL and VLDL. After filtration to remove the latex beads, LDL is left in the supernatant and its cholesterol content is measured. That procedure requires several expensive antibodies to adsorb HDL and VLDL.

LDL may be isolated from other lipoproteins by agglutinating the LDL with a lectin. Agglutination is a clumping together of LDL particles which causes them to precipitate. Therefore, the cholesterol content of isolated LDL may be determined (Sears, U.S. Pat. No. 4,190,628).

Recently, a method for the direct quantitative determination of LDL cholesterol in a sample of blood plasma was proposed (U.S. Pat. Nos. 4,883,765 and 5,141,872). It involves selective adsorption of lipoproteins on silica, removal of HDL by incubation in a suitable detergent solution, extraction of the remaining LDL cholesterol by another deterg nt and d termination of the LDL cholesterol by spectrophotometric analysis. However, that proc ss is also very time consuming.

Consequently, there is a need for a simple, rapid, inexpensive and easy to operate procedure and/or device for the determination of lipoprotein cholesterol.

SUMMARY OF THE INVENTION

It is the object of the present invention to provide an assay procedure and/or device which permits direct measurement of VLDL, LDL, HDL as well as apolipoproteins cholesterol in blood plasma.

The present invention provides a method for direct quantitative determination of VLDL, LDL, HDL as well as apolipoproteins cholesterol in a sample of blood plasma comprising the following steps in which:

a) An antibody against the lipoprotein or apolipoprotein is partitioned in one of the phases of aqueous two-phase system with or without the help of partitioning enhancer by conjugation;

b) A sample of blood plasma is applied to an aqueous two-phase system;

c) After mixing and incubation for a short period of time, the phases are separated by gravity or centrifugation within several minutes;

d) The phase containing the antibody and the targeted lipoprotein or apolipoprotein is removed;

e) The targeted lipoprotein cholesterol is directly determined with an enzymatic procedure.

These and further and other objects and features of the invention are apparent in the disclosure, which includes the above and ongoing written specification, with the claims.

DETAILED DESCRIPTION OF THE INVENTION

This invention is based on an aqueous two phase system in which plasma cholesterols are partitioned in the bottom phase while polyethylene glycol (PEG) conjugated anti-LDL monoclonal antibody (PEG-Mab) is partitioned in the top phase. PEG-Mab helps specifically the LDL transportation to the top from the bottom phase. Plasma LDL cholesterol value is obtained by measuring the top phase cholesterol.

The aqueous two-phase systems ar formed when two polym rs, such as PEG and dextran, or one polymer and one low molecular weight component (e.g. salt), such as PEG and potassium phosphate, are mixed at appropriate concentrations in the presence of water. The polymers are generally water-soluble. In these systems, the distribution of a protein between the phases depends on the properties of respective phases and the protein. The conditions of the system is adjustable so that lipoprotein or apolipoprotein is partitioned in one phase (for example the bottom phase). The addition of polyethylene glycol conjugated with an antibody against LDL will bring only LDL to another phase (i.e. the top phase). The measurement of cholesterol of that phase (top phase) will therefore give the plasma LDL cholesterol value.

The components of the first phase of the aqueous two-phase system may be selected from polyethylene glycol, polyvinyl alcohol, polypropylene glycol, dextran, etc. and components for the second phase may be selected from dextran, methyl cellulose, potassium phosphate, etc. The specific reagents used to adsorb the lipoproteins or apolipoproteins are preferable monoclonal antibodies of the lipoproteins or apolipoproteins. But certainly polyclonal antibodies may also be used wherever possible for purely economical reasons. By standard procedures, such as those developed by Harris, et al. in J. Polymer Science, 22, 341 (1984), these antibodies may be readily conjugated to the selected components of the first phase. Because of the specific functions of these conjugate components, they are termed as the partitioning enhancers or anchoring agents in the present invention.

The present invention is simple, rapid, inexpensive, and easy to perform. One of the great advantages is that only one antibody is used. It is also easily applicable to other lipoprotein cholesterol measurements such as lipoprotein (a) [Lp(a)], HDL and VLDL. It can even be applied to measure the cholesterol of denatured lipoproteins, such as malondiald hyde-reacted LDL (Kondo, et. al. E. P. 484,863) or glycated LDL

(Cohen, WO 94/00592), or the cholesterol of apolipoproteins, such as A-I, A-II, A-IV, B-48, B-100, C-I, C-II, C-III, D and E (Albers, et al., Clinics in Laboratory Medicine, 137, 1989). All of these lipoprotein cholesterols are measurable with the same approach as the present invention, but with different antibodies according to the targeted lipoprotein or apolipoprotein.

Other features of this invention will become apparent in the following description of exemplary embodiments which are given for illustration of the invention and not intended to be limiting thereof.

Examples

Example 1: Preparation of Aqueous Two-Phase System

Several different phase systems were tested in order to find an ideal one for this invention. In PEG 3,400/ dextran T-70 and PEG 3,400/Ficoll 400,000 systems, lipoproteins are exclusively partitioned in the bottom (dextran-rich or Ficoll-rich) phase. However, addition of PEG-anti-LDL conjugate could not bring LDL to the PEG phase, probably due to high viscosity of dextran or Ficoll. In PEG 1,000/phosphate, proteins precipitated. PEG 8,000/phosphate and PEG 3,400/phosphate was found to be best in which lipoproteins partitioned in the bottom phase and addition of PEG-anti-LDL brought LDL to the top phase. Because PEG 8,000 has higher viscosity, PEG 3,400 was therefore chosen in further study.

PEG 3,400 and phosphate phase system was prepared according to the following composition:

7.93 wt% K_2HPO_4, 4.36 wt% KH_2PO_4, 13.7 wt% PEG 3,400 and 74.01 wt% H_2O at pH=7.0.

Example 2: Cholesterol Measurement of Lipoprotein or Plasma

There are several available methods for determination of cholesterol (Tanks, Clin. Biochem., 1,12, (1967)). Enzymatic measurement is most commonly used in clinical laboratory because it is fast, accurate and easily automated. Sigma Diagnostic Kit 352 (Sigma Chemical, St. Louis, MO) measures total cholesterol level nzymatically, which is a modification

of the method of Allain t al., Clin. Ch m., 20, 470 (1974). It m asures both free and st rifi d cholest rol. Th procedure used in this invention is briefly described as follows. Human plasma, LDL, HDL, VLDL, cholesterol standard or the selected portion of the two-phase system were added to the cholesterol reagent. After standing for 15 minutes at room temperature, the absorbances at 500nm were measured and cholesterol levels were calculated based on the absorbance of cholesterol standard which is certified for use with Sigma Kit 352 by CDC and NCEP.

Example 3: Partitioning of LDL, VLDL and HDL

Ultracentrifuge prepared LDL was used to examine the partitioning of LDL in the two-phase system. In the PEG 3,400/phosphate system described in Example 1, LDL was found to partition in the bottom phase, as measured by the cholesterol contents. Ultracentrifuge prepared HDL and VLDL were also virtually partitioned in the bottom phase.

Example 4: Partitioning of Human Plasma

When human plasma was partitioned in the two-phase system described in example 1, the majority but not all of the cholesterols was found in the bottom phase. Addition of acid to the two-phase system leads to more cholesterol partitioned in the bottom and much less in the top. Alkaline has the reverse effect.

Cholesterol was virtually found in the bottom phase when human plasma was placed in the two phase solution with addition of 40 μl of 1 N HCl to 1 ml two-phase system.

Example 5: Conjugation of Monoclonal Antibody Anti-LDL-apo B to PEG

A monoclonal antibody against T2 fragments of LDL apo B (Caltag, So. San Francisco, CA) binding specifically to LDL and not cross-reacting with VLDL and Lp(a) was used in this preparation. 600 μl of monoclonal antibody solution with 1 mg/ml in 0.1 M $NaHCO_3$ was added to 100 μl of methoxy-poly thylene glycol nitrophenyl carbonate (Sigma chemical, St. Louis, MO). The mixture was incubated for 24 hours at 4°C.

The conjugated material was stored at 4°C for furth r us .

Example 6: Partitioning of PEG-Anti-LDL-apo B in Two-phase System

The antibody of LDL itself is hydrophilic and partitions in the phosphate phase of PEG/phosphate system. However, when that antibody is attached to a hydrophobic molecule like PEG, the conjugated molecules, PEG-antibody, partitions in the PEG phase. Here the conjugated PEG is called partitioning enhancer or anchoring agent in the present invention.

The PEG-anti-LDL-apo B conjugate or simply PEG-Mab was partitioned in the two-phase system described in Example 1. Solution from each phase (after dilution with PBS) were added to ELISA plate coated with LDL. Alkaline phosphatase conjugated goat anti-mouse IgG was used for the ELISA reading detection. The result showed that PEG-Mab partitioned in the top phase.

Example 7: LDL Cholesterol Determination Using Beta-Quantification

Plasma samples were ultracentrifuged at 50,000 RPM for 18 hours at 10°C. The top VLDL fraction was removed and the bottom HDL-LDL infranant was collected. The bottom of the centrifuge tube was washed with 0.15 M NaCl and the solution and infranant were combined. 40 µl of 5,000 unit/ml heparin (Sigma, Cat. No. H9399) was added to 1 ml of HDL-LDL infranant and mixed. Then 50 µl of 1 M $MnCl_2$ was added and mixed by vortexing. After standing for 30 min on ice, the mixture was centrifuged for 30 min at 1,500 g at 4°C. LDL was precipitated and HDL was left in the supernatant. The cholesterol contents of HDL-LDL fraction and that of the HDL in supernatant were measured. The difference gave the LDL cholesterol.

Example 8: Partitioning of LDL and Plasma in the Immunoaffinity Aqueous Two-Phase System

10 µl of plasma or 1 µl of LDL standard sample was incubated with 10 µl of PEG-Mab for 1 hour and then transferred to 200 µl of aqueous two-phase system (100 µl each phase). 50 µl of each phase was added to 667 µl of cholesterol reagent for cholesterol m asurement, LDL cholesterol was calculated from

the cholesterol content of the top phase. The reading thus obtain d was termed as LDL cholesterol by two-phase measurement. Ten human plasma samples were evaluated for their LDL cholesterol level by both beta-quantification and by aqueous two-phase system. The comparison is shown in Table 1. While both readings may fall in reasonably close range, yet steady readings are made possible by improving the partition coefficient of PEG-Mab in aqueous two-phase system, as well as their conjugation chemistry.

Table 1

Comparison of LDL Cholesterol by Beta-Quantification and Aqueous Two-Phase System

Sample	Total Cholesterol mg/dl	LDL Cholesterol Beta-Quantification	LDL Cholesterol Aqueous Two Phase System
1	171	84	75
2	140	66	53
3	121	62	59
4	118	54	55
5	128	58	65
6	156	75	70
7	138	76	66
8	116	56	59
9	110	61	61
10	139	79	74

Obviously, numerous modifications and variations of the present invention are possible in the light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

While the invention has been described with reference to specific embodiments, modifications and variations of the invention may be constructed without departing from the scope of the invention, which is defined in the following claims.

I claim:

1. (Amended) A method for quantitative determination of the cholesterol value of lipoprotein or apolipoprotein from body fluid, comprising the steps of:

a) preparing an aqueous two-phase system comprising at least two water-soluble polymers;

b) preparing antibody conjugates by coupling antibodies of a targeted lipoprotein or apolipoprotein to partitioning enhancers to improve a partitioning of conjugates of one of said two phases;

c) adding body fluid to the antibody conjugates, mixing to form a mixture of the body fluid and the antibody conjugates and incubating for a period of time sufficient to form a specific binding complex composed of targeted lipoprotein or apolipoprotein and antibody conjugates if any;

d) applying the mixture consisting of the body fluid and the antibody conjugates to the aqueous two-phase system;

e) after mixing and incubating sufficiently, separating the two phases by gravity or centrifuge;

f) removing one of said two phases containing the antibody conjugates;

g) directly determining the cholesterol content of said removed phase.

2. The method as claimed in claim 1, wherein the partition enhancer is polyethylene glycol or its derivative.

3. The method as claimed in claim 1, wherein the lipoprotein is low density lipoprotein.

4. The method as claimed in claim 1, wherein the antibody of the targeted lipoprotein has a high partition coefficient to the phase one with or without coupling to a partitioning enhancer.

5. (Amended) A method for quantitative determination of the cholesterol value of lipoprotein or apolipoprotein from body fluid, comprising the steps of:

a) preparing an aqueous two-phase system comprising at least one-water-soluble polymer and at least one low molecular

weight water-soluble component, in amounts sufficient to form said aqueous two-phase system;

b) preparing antibody conjugates by coupling antibodies of a targeted lipoprotein or apolipoprotein to partitioning enhancers to improve a partitioning of conjugates of one of said two phases;

c) adding body fluid to the antibody conjugates, mixing to form a mixture of the body fluid and the antibody conjugates and incubating for a period of time sufficient to form a specific binding complex composed of targeted lipoprotein or apolipoprotein and antibody conjugates if any;

d) applying the mixture consisting of the body fluid and the antibody conjugates to the aqueous two-phase system;

e) after mixing and incubating sufficiently, separating the two phases by gravity or centrifuge;

f) removing one of said two phases containing the antibody conjugates;

g) directly determining the cholesterol content of said removed phase.

6. The method as claimed in claim 5, wherein the partition enhancer is polyethylene glycol or its derivative.

7. A method as claimed in claim 5, wherein the lipoprotein is low density lipoprotein.

8. A method as claimed in claim 5, wherein the antibody of the targeted lipoprotein has a high partition coefficient to the phase one with or without coupling to a partitioning enhancer.

9. A method for quantitative determination of the cholesterol value of lipoprotein or apolipoprotein from body fluid, comprising the steps of:

a) preparing an aqueous two-phase system comprising at least two water-soluble polymers;

b) preparing antibody conjugates by coupling antibodies of a targeted lipoprotein or apolipoprotein to partitioning enhancers to improve a partitioning of conjugates of one of said two phases;

c) adding a sample to be test d, and the antibody conjugates to the aqueous two-phase system;

d) after mixing and incubating, separating the two phases by gravity or centrifuge;

e) removing one of said two phases containing the antibody conjugates;

f) directly determining the cholesterol content of said removed phase.

10. A method for quantitative determination of the cholesterol value of lipoprotein or apolipoprotein from human plasma or body fluid, comprising the steps of:

a) preparing an aqueous two-phase system comprising at least one water-soluble polymer and at least one low molecular weight water-soluble component, in amounts sufficient to form said aqueous two-phase system;

b) preparing antibody conjugates by coupling antibodies of a targeted lipoprotein or apolipoprotein to partitioning enhancers to improve a partitioning of conjugates of one of said two phases;

c) adding a sample to be tested, and the antibody conjugates to the aqueous two-phase system;

d) after mixing and incubating, separating the two phases by gravity or centrifuge;

e) removing one of said two phases containing the antibody conjugates;

f) directly determining the cholesterol content of said removed phase.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CLINICS IN LABORATORY MEDICINE, Volume 9, Number 1, issued March 1989, Bachorik, "Measurement of Total Cholesterol, HDL-Cholesterol, and LDL-Cholesterol", pages 61-72, see pages 64-65, 69-70.	1-10
Y	US, A, 4980,065 (HSU) 25 December 1990, see columns 1, 7 and 8.	1-10
Y	US, A, 5,078,886 (HSU) 07 January 1992, see columns 1, 8 and 9.	1-10
Y	US, A, 4,746,605 (KERSCHER ET AL.) 24 May 1988, see column 2, line 40 - column 3, line 6.	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search 02 OCTOBER 1995	Date of mailing of the international search report 24 OCT 1995
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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,945,040 (FLESS ET AL.) 31 July 1990, see column 2, lines 5-8.	1-10
Y	EP, A, 0,484,863 (DAIICHI PURE CHEMICALS CO.) 13 May 1992, see page 2, lines 5-8.	3, 7
A	US, A, 5,093,254 (GIULIANO ET AL.) 03 March 1992, see entire document.	1-10
A	US, A, 5,407,810 (BUILDER ET AL.) 18 April 1995, see entire document.	1-10
A	JOURNAL OF CHROMATOGRAPHY, Volume 513, issued 1990, Diamond et al., "Correlation of protein partitioning in aqueous polymer two-phase systems", pages 137-143, see entire document.	1-10
A	JOURNAL OF IMMUNOLOGICAL METHODS, Volume 38, issued 1980, Mattiasson et al., "Partition Affinity Ligand Assay (PALA) A New Approach to Binding Assays", pages 217-223, see entire document.	1-10

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER:
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A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/7.1, 7.92, 11; 436/71, 536, 547, 548; 210/632

B. FIELDS SEARCHED
Minimum documentation searched
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435/7.1, 7.92, 11, 962; 436/71, 536, 547, 548, 824, 825; 210/632

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG
search terms: lipoprotein, apolipoprotein, cholesterol, phase, partition, antibody, polyethylene glycol